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| 2. Patent application number *(The Patent Office will fill in this part)* | **9902659.3** |
| 3. Full name, address and postcode of the or of each applicant *(underline all surnames)* | Microbiological Research Authority<br>CAMR<br>Porton Down<br>Salisbury<br>Wiltshire SP4 0JG |
| Patents ADP number *(if you know it)* | 6566160001 |
| If the applicant is a corporate body, give the country/state of its incorporation | UK |
| 4. Title of the invention | ASSAY WITH REDUCED BACKGROUND |
| 5. Name of your agent *(if you have one)* | |
| "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* | MATHYS & SQUIRE<br>100 Grays Inn Road<br>London WC1X 8AL |
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# ASSAY WITH REDUCED BACKGROUND

The present invention relates to an assay with reduced background, a method of assaying for an analyte, a method of reducing background in an assay and apparatus, in particular a test kit, for carrying out such an assay.

ATP bioluminescence has rapidly become the method of choice for hygiene and cleanliness monitoring due to its combination of sensitivity and ease of assay. A luciferin-luciferase bioluminescence assay can detect as little as $10^{-15}$ moles of ATP. Since an average microbial cell contains approximately $10^{-18}$ moles of ATP, this gives a detection limit of only $10^{3}$ cells.$ml^{-1}$.

For most operations this detection level is sufficient, however, there are applications where even greater sensitivity is required, even down to a single microbial cell. GB-A-2304892 describes such an assay using the ATP-forming enzyme adenylate kinase (AK). An average cell contains several hundred-fold less AK molecules than ATP molecules, however, in a 10 minute incubation, a typical 400,000-fold amplification is achieved by detecting AK through the ATP it produces. This corresponds to the level of single cell detection, although in practice 10 cells.$ml^{-1}$ is more readily achieved due to background AK and ATP contamination. It also corresponds to a detection level of down to at least $10^{-20}$ moles of AK.

The commercial use of this extreme sensitivity is, therefore, under investigation. There are, however, some problems with more widespread use of this known AK-based assay. One is that while the assay detects the presence of micro-organisms, it does not differentiate between one organism and another. This has been overcome to a degree by the use of bacteriophage to release AK from specific bacteria (Blasco R, Murphy MJ, Sanders MF and Squirrell DJ (1998) Specific assays for bacteria using phage

mediated release of adenylate kinase. *J.Appl.Microbiol.* 84: 661-666).

Each micro-organism, however, requires a specific phage and contains an AK with different buffer requirements, plus temperature and pH optima. The second problem is more fundamental and is a problem for its use as a generalised reporter enzyme. Whereas in hygiene and cleanliness monitoring the ubiquity of ATP and AK is beneficial, in an enzyme reporter assay any unwanted background activity is detrimental. This is especially so where the sample is greatly concentrated to maximise potential detection.

A further problem is that the known assay is only effective for microorganisms which contain AK; the known assay will not work with other biological material, such as viruses or other analytes, including other biological such material that does not contain AK.

The highest native concentrations of TSE infectivity are found in 263K scrapie-infected hamster brain where titres as high as $10^{10}$ infectious units per gram of tissue are frequently reported. Such brains have been shown to contain approximately 100 g of TSE-specific amyloid per gram of tissue, which implies an infectivity for pure scrapie amyloid of approximately $10^{14}$ infectious units per gram, but this is not found in practice due to losses during purification.

Scrapie amyloid has been shown to contain predominantly, if not exclusively, an aggregated form of $PrP^{Sc}$. Scrapie infectivity (and *in vitro* $PrP^{C}$ converting activity) has been shown to lie with residual, partially protease-resistant, multimers of $PrP^{Sc}$ rather than the $PrP^{Sc}$ monomer. $PrP^{Sc}$ has a molecular weight in the range 33-35kD and contains a protease-resistant core of 27-30kD (PrP27-30). One gram of scrapie amyloid, therefore, contains approximately $2 \times 10^{19}$ molecules of $PrP^{Sc}$, giving a single infectious unit corresponding to $2 \times 10^{5}$ molecules of $PrP^{Sc}$ ($\sim 3.4 \times 10^{-19}$ moles). Any method for detecting PrP must, therefore, aim for a level of

sensitivity corresponding to below this limit.

Current immunoassays give positive signals for $PrP^{Sc}$ from as little as 1-10 g of TSE infectious brain tissue, e.g. by Western blotting or ELISA. ELISA, however, is considerably more suitable than Western blotting for the development of a fast and practical PrP ($PrP^{C}$ + $PrP^{Sc}$) detection system. By analogy from the above, this level of detection is approximately $10^{-14}$ moles of $PrP^{Sc}$ and, therefore, insufficient to detect the presence of still infectious quantities of $PrP^{Sc}$.

Where $PrP^{C}$ is also included, however, the differential between the current and required level of sensitivity is significantly reduced. This brings current immunoassays potentially into the appropriate range, but with an inadequate margin of safety.

Therefore, it remains a problem to provide an assay for biological material, especially prior protein, of increased sensitivity.

The decontamination of any surface or object exposed to TSE infectivity comprises two elements: cleaning and disinfection. The presence of prion protein ($PrP^{C}$ and/or $PrP^{Sc}$) is presently detected most sensitively by immunoassay (e.g. Western blot or ELISA) while infectivity is best identified by *in vivo* bioassay. *In vivo* bioassay is a prolonged and expensive procedure and, therefore, unsuitable for the routine validation of any decontamination regime. Immunoassay is more rapid but not a direct indicator of TSE infectivity. It is possible to envisage that a proposed TSE inactivation procedure, whether chemical (e.g. sodium hydroxide), physical (e.g. autoclaving at 134-138°C) or biological (e.g. thermoproteolysis) might be successful in eliminating TSE infectivity but leave $PrP^{C}$ or $PrP^{Sc}$-derived material still capable of eliciting a reaction with PrP antibodies.

A PrP immunoassay, however, can be validated by *in vivo* bioassay to show

whether it has sufficient sensitivity, i.e. a negative result corresponds to below the level of detectable TSE infectivity. Monitoring of a validated cleaning procedure rather than a disinfection procedure, therefore, offers the better prospect for routine determination of decontamination. Since only cleanliness is being monitored directly, the immunoassay need not discriminate between $PrP^{C}$ and $PrP^{Sc}$ and can, therefore, be based upon the detection of total PrP. This offers an additional safety margin since even in a worst case scenario (e.g. a microtome blade used to section a heavily infected brain), infective $PrP^{Sc}$ will only be a fraction of total PrP.

There is currently great uncertainty regarding the numbers of individuals in the UK potentially or actually infected with new variant Creutzfeld-Jakob Disease (nvCJD). As a result there have been calls that all surgical procedures should be carried out using disposable instruments as a safeguard. Implementation has severe cost and procedural implications, consequently an alternative means to validate decontamination would be extremely beneficial, and would also be of benefit to other equipment such as meat processing equipment.

The present invention is aimed at addressing and overcoming or at least ameliorating these problems.

A further object of specific embodiments of the present invention is to develop a rapid and sensitive method for the detection of prion protein PrP ($PrP^{C}$ and $PrP^{Sc}$) - as the presence of either isoform in a sample is indicative of the presence of residual PrP-expressing tissue and the potential for transmissible infectivity.

A still further object of specific embodiments of the present invention is to provide a method for assay of prion proteins that may be used in the screening of cleaning protocols to determine their suitability for the removal of TSE agents from surfaces and delivery of recovered material for

immunoassay.

Accordingly, a first aspect of the invention provides an assay for an analyte, comprising specifically associating the analyte with a reporter adenylate kinase, adding ADP and testing for formation of ATP wherein, prior to addition of ADP, adenylate kinase other than reporter adenylate kinase is substantially removed.

Thus, in the assay of the present invention, the reporter adenylate kinase is specifically associated with the analyte so that the amount of reporter adenylate kinase is substantially in proportion to the amount of analyte present. In the absence of analyte there will be no reporter adenylate kinase associated and no signal generated. By substantially removing adenylate kinase other than reporter adenylate kinase, the present invention has the advantage that the signal obtained is not contaminated or otherwise adversely affected by any endogenous adenylate kinase that might have been present in a sample being tested. By reference to removing adenylate kinase it is intended to refer to removing adenylate kinase activity, such as by removing the adenylate kinase, or denaturing or otherwise inactivating it in situ. Furthermore, by addition of reporter adenylate kinase, the assay is of application for detection of substantially any analyte and, unlike the prior art, is not limited to detecting analytes that comprise their own adenylate kinase.

In an embodiment of the invention there is provided a method of determining presence and/or amount of an analyte in a sample, comprising:-

> exposing the sample to a reporter adenylate kinase coupled to a binding agent specific for the analyte, so that the reporter adenylate kinase is specifically associated with any analyte present in the sample;

removing reporter adenylate kinase that is not specifically associated with analyte;

exposing reporter adenylate kinase specifically associated with the analyte to ADP; and

testing for formation of ATP,

wherein prior to addition of ADP adenylate kinase other than reporter adenylate kinase is substantially removed.

Typically, the reporter adenylate kinase is coupled to an antibody that binds specifically to the analyte under investigation. The antibody may be obtained using conventional techniques for identification and isolation of specific antibodies, and the assay of the present invention is thus of application to substantially all analytes against which an antibody can be raised. This confers the advantage that the present invention is of considerably wider application compared to the known AK/ATP - based assays, as the previous assays were restricted to target analytes that contained their own adenylate kinase.

The reporter adenylate kinase is coupled to the specific binding agent by conventional techniques. For example, there are numerous ways of labelling immunoreactive biomolecules with enzymes (conjugation). Antibodies, the majority of antigens, and enzymes are all proteins and, therefore, general methods of protein covalent cross-linking can be adapted to the production of immunoassay reagents, The preparation of antibody-enzyme conjugates requires mild conditions to ensure the retention of both the immunological properties of the antibody and the catalytic properties of the enzyme. Common methods include, glutaraldehyde coupling, the use of periodate oxidation of glycoproteins to generate dialdehydes capable of forming Schiff-base linkages with free amino groups on other protein molecules, and the

use of heterobifunctional reagents, for example, succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC).

Endogenous adenylate kinase present in the analyte is substantially removed or destroyed or otherwise inactivated before testing for formation of ATP is carried out. This removal step can conveniently be achieved by heating the endogenous adenylate kinase to a temperature at which it is denatured. Alternatively, other treatments might be appropriate to destroy the activity of the endogenous adenylate kinase, such as the use of ultrasound or extremes of pH or salt concentration. In an embodiment of the invention, the reporter adenylate kinase is a thermostable enzyme and endogenous adenylate kinase is removed by heating. In a specific embodiment of the invention described in more detail below, this denaturing step is carried out at about 90°C for a period of about 10 minutes, though other temperatures and durations will be appropriate so long as the endogenous adenylate kinase is rendered incapable of catalysing the formation of ATP and the reporter adenylate kinase retains its activity.

It is a further, preferred, step in the assay of the present invention for any ATP present prior to addition of ADP to be removed, thereby further decreasing the background noise in the assay. The removal of endogenous ATP may be achieved by addition of an ATPase and incubation prior to adding ADP. More preferably, a thermolabile ATPase is used to remove ATP and then the thermolabile ATPase is itself destroyed by use of elevated temperature, to avoid the presence of the ATPase adversely influencing the signal obtained using the thermostable, reporter adenylate kinase.

The precise order of carrying out the steps of the present invention is not critical, provided that endogenous adenylate kinase is destroyed before addition of ADP and testing for the formation of ATP. Thus, the method of the present invention can be carried out by treating a sample to destroy its endogenous adenylate kinase, adding reporting adenylate kinase coupled to

an antibody specific to the analyte, isolating reporting adenylate kinase that is specifically associated with analyte and then adding ADP and testing for formation of ATP. Alternatively, the assay can be carried out by adding a reporter adenylate kinase coupled to an antibody specific for the analyte to a sample, isolating reporter adenylate kinase that is specifically associated with analyte, destroying any endogenous adenylate kinase that may be present and then adding ADP and testing for formation of ATP. A further alternative is to add reporter adenylate kinase coupled to an antibody specific for analyte to the sample, treating the sample to destroy endogenous adenylate kinase, isolating reporter adenylate kinase specifically associated with analyte and then adding ADP and testing for formation of ATP.

In a specific embodiment of the invention described in more detail below, an assay is carried out by following the steps:-

1. An antibody specific to the analyte is immobilised on a solid phase.

2. A sample is combined with the solid phase so that analyte present in the sample can bind to the antibody.

3. The solid phase is washed, thereby washing away components of the sample and retaining on the solid phase only any analyte that has bound to the immobilised antibody.

4. A reporter composition is added to the solid phase, the reporter composition comprising an antibody which is specific to the analyte and which is coupled to a thermostable adenylate kinase.

5. The solid phase is washed, thereby washing away unbound

components of the reporter composition and retaining reporter composition that has specifically bound the analyte, the analyte being itself bound to the immobilised antibody.

6. The solid phase is heated to denature any endogenous adenylate kinase that may be present but so as not to denature the thermostable adenylate kinase.

7. Optionally, a thermolabile ATPase is added to the solid phase to remove any endogenous ATP.

8. Optionally, the solid phase is heated to destroy the thermolabile ATPase of step 7.

9. ADP is added to the solid phase which is then tested for presence and/or amount of ATP.

10. If ATP is detected, this indicates that adenylate kinase in the reporter composition was bound to the solid phase, ie that analyte was present in the sample.

The solid phase is suitably selected from conventional solid phases used in immunoassays, and can for example be a microtitre well, a column, a dip-stick or a bead, such as a latex or a magnetic bead. Examples of further suitable solid supports are nitrocellulose, polyvinylchloride, polystyrene, diazotized paper, activated beads having a range of appropriate linking agents and S.aureus protein A beads. More thermostable supports are provided by plastics such as polypropylene, polycarbonate, polyphenylenine oxide polymethylpentene and fluoropolymers (e.g. PTFE, PFA, FEP and EFTE). The solid support can have several forms dependent upon the type of support and the conditions required. Commonly these will be microtitre plates, where each individual well serves as an independent incubation

chamber. Similarly, membranes or sheets can be used providing lateral diffusion is limited. Alternatively, beads can be used, which enable the separate reactions to be performed in different tubes under different conditions. These individual matrix materials can be purchased in a variety of forms, as appropriate for the particular type of assay.

Firefly luciferin catalyses the oxidation of D(-) luciferin in the presence of ATP-$Mg^{2+}$ and $O_2$ to generate oxyluciferin and light. The quantum yield for this reaction (0.88) is the highest known for bioluminescent reactions (Gould and Subramini, 1988). Firefly luciferase, however, is relatively unstable and has, therefore, not proved readily adaptable as an immunoassay label (Kricka, 1993). By contrast, in the present invention, the luciferase enzyme can be operated under its optimal conditions and is not exposed to harsh treatments such as antibody-coupling.

A number of extremely thermostable adenylate kinases have now been characterised (Ki and Takahisa, 1988; Lacher and Schäfer 1993; Rusnak *et al.*, 1995) and are suitable for use in the present invention. One has been cloned and overexpressed in *E.coli* (Bonisch *et al.*, 1996) and the full sequences of a range of others are now available as a result of genome sequencing programmes. A rapid and simple purification scheme is thus available to produce homogenous adenylate kinase. Initially a thermal denaturation step can be employed to denature the bulk of *E.coli* proteins (~90-95%) while retaining the thermostable activity in solution.

This procedure has been successfully employed in embodiments of the present invention with several recombinant thermostable enzymes. Subsequently a generally applicable affinity purification procedure can be utilised to yield the purified enzyme. This involves binding of the enzyme to a mimetic dye matrix and selective desorption with the adenylate kinase inhibitor $P^1$, $P^5$-di (adenosine-5′) pentaphosphate (Rusnak *et al.*, 1995). The use of stable enzymes should overcome any problems associated with

inactivation upon antibody-coupling, but also provide other benefits. Since the activity is extremely thermostable, once substrate binding and removal of unbound components has occurred, the temperature can be increased to e.g. 70-90°C, denaturing and inactivating any residual contaminating mesophilic adenylate kinase. Additionally, on cooling, a mesophilic ATPase (or apyrase) can be added to remove any residual ATP. This ensures that no ATP or AK background is now present. A further heat incubation inactivates the mesophilic ATPase and ADP is added in order to generate ATP derived exclusively from the thermostable adenylate kinase. This ATP is then available for conventional luciferin-luciferase bioluminescence detection. A potentially contaminating ATP signal is now only possible from three sources: non-specifically bound thermostable AK, ATP-contaminated ADP and AK contaminated luciferase. The latter two can be eliminated by the use of high purity reagents and careful handling. In each case, however, contamination would result in a positive signal, i.e. a PrP-free sample might be determined to be PrP-containing but the opposite could not occur.

A known thermostable adenylate kinases, *Methanococcus jannaschii* has a very high specific activity, namely 89 $\mu$mol of ATP $mg^{-1}$ $min^{-1}$. This corresponds to a turnover number in excess of 2000 $min^{-1}$ and the potential to produce more than $1.2 \times 10^5$ molecules of ATP per molecule of AK in an hour's incubation. Since $6 \times 10^8$ molecules of ATP are detectable by ATP-bioluminescence then as few as $5 \times 10^3$ molecules of PrP would be detectable. This is 40-fold lower than the minimum number of $PrP^{Sc}$ molecules identified as constituting a single infectious unit. An additional safety margin is provided by the presence of much higher quantities of $PrP^C$ in relation to $PrP^{Sc}$ indicating that the present invention exceeds the required sensitivity by several orders of magnitude.

As an alternative to use of an analyte-specific antibody to immobilize analyte on the solid phase, the solid phase may be provided with analyte immobilised directly thereon without the presence of the first antibody. For

example, the solid phase can itself be a substrate potentially contaminated by an amount, typically a trace amount, of analyte. This is the case in respect of medical equipment potentially contaminated by very small amounts of prion protein which are effectively immobilised on the surface of the equipment. The assay is of use in testing for the presence of the analyte for example following cleaning of the equipment. Analyte can also be immobilised non-specifically.

The method of the present invention may be carried out utilising relatively inexpensive equipment in a standard laboratory. Use of a method of the present invention to determine when the level of prion protein has been reduced to below detectable and, by extrapolation, infectious levels may be used to confirm the decontamination of instruments, equipment and other items potentially exposed to TSE infectious agents, permitting their safe use.

In use of a specific embodiment of the invention, the first washing step can be repeated a number of times, in accordance with conventional practice in this field, the object being to remove from the solid phase all components of the sample that have not bound specifically to the immobilised antibody. Thus, if there is no analyte present in the sample then the washing step will remove the whole of the sample and ultimately the assay will give no signal, indicating that no analyte was present. The antibody in the reporter composition binds to the same analyte as the antibody immobilised on the solid phase. The antibody and the reporter composition can in fact have the same binding properties as the immobilised antibody, though it is an alternative for the reporter antibody to bind to a different site on the same analyte. The reporter antibody is preferably selected so that the amount of reporter composition that binds to the analyte is substantially proportional to the amount of analyte present. The second washing step can, in line with the first, be repeated a number of times in accordance with conventional practice, the object of the second washing step being to remove all components of the reporter composition that have not specifically bound to

analyte which itself has specifically bound to immobilised antibody. Thus, if no analyte is present on the solid phase the second washing step is to remove all reporter composition, leading ultimately to no signal being generated in the assay, indicating no analyte was present in the sample under investigation.

This latter embodiment represents use of the principles of the invention in a two antibody capture assay, sometimes referred to as a sandwich assay. The invention is similarly of application in antigen capture assays and antibody capture assays.

Thus in a further embodiment of the invention, an assay for analyte comprises specifically associating an analyte with a reporter adenylate kinase, wherein the analyte is bound to a solid phase. This embodiment may be referred to as being of the antibody capture type. Binding of the analyte to the solid phase can be achieved by non-specifically binding the analyte to the solid phase and then treating the solid phase to prevent further non-specific binding thereto - in this way, a number of components from a sample are bound to the solid phase, which components include the analyte of interest if present in the sample, and subsequent treatment ensures that when an antibody is added to detect the analyte that antibody will only bind to the solid phase if analyte is present.

The use of heat to denature any endogenous adenylate kinase that may be present has been carried out in an embodiment above as step 6, though as mentioned this step can be carried out at an alternative juncture in the assay provided that it is carried out before addition of ADP. Further, ADP may be added before the ATPase provided the ATPase has no ADPase activity. The temperature and duration adopted are chosen so as to be sufficient to denature the endogenous adenylate kinase whilst leaving intact the reporter adenylate kinase, this reporter adenylate kinase preferably being a thermostable enzyme. In a specific embodiment described below, heating

to a temperature of about 90°C for about 10 minutes has been found effective. Sufficiently thermostable adenylate kinases may be found amongst a range of bacterial and archaeal genera and families. In the Bacteria, they may be produced, for example, by members of the genera *Alicyclobacillus, Ammonifex, Aquifex, Bacillus, Caldariella, Calderobacterium, Caldicellulosiruptor, Caldocellum, Caloramator, Carboxydothermus, Chloroflexus, Clostridium, Coprothermobacter, Dictyoglomus, Fervidobacterium, Geotoga, Hydrogenobacter, Hydrogenothermophilus, Meiothermus, Petrotoga, Rhodothermus, Rubrobacter, Thermoactinomyces, Thermoanaerobacter, Thermoanaerobacterium, Thermoanaerobium, Thermobacterium, Thermobacteroides, Thermobifida, Thermobispora, Thermobrachium, Thermochromatium, Thermocrispum, Thermodesulfobacterium, Thermodesulforhabdus, Thermodesulfovibrio, Thermohydrogenium, Thermomicrobium, Thermomonospora, Thermonema, Thermonospora, Thermopolyspora, Thermosipho, Thermosphaera, Thermosyntropha, Thermoterrabacterium, Thermotoga* and *Thermus*. Amongst the archaea, they may be produced, for example, by members of the genera *Acidianus, Aeropyrum, Archaeoglobus, Desulfurococcus, Desulfurolobus, Ferroglobus, Hyperthermus, Metallosphaera, Methanobacterium, Methanococcus, Methanopyrus, Methanothermus, Picrophilus, Pyrobaculum, Pyrococcus, Pyrodictium, Pyrolobus, Staphylothermus, Stetteria, Stygiolobus, Sulfolobus, Sulfophobococcus, Thermococcus, Thermofilum, Thermoplasma* and *Thermoproteus*.

It is preferred, though optional, also to carry out a step of removing endogenous ATP from the sample using a thermolabile ATPase and subsequently destroying this latter enzyme, again conveniently using heat. In a-specific embodiment of the invention described.below, an incubation of about 10 minutes has been effective using a thermolabile ATPase and this enzyme has been then denatured by temperatures of about 90°C for 5 minutes. ATP can be released from cells or other cellular components after

heating. Therefore, it is preferred that the step of removing ATP is carried out after an initial heating of the sample, for example after the step of using heat to destroy endogenous adenylate kinase.

The present invention also provides, in a second aspect, apparatus for determining the presence and/or amount of analyte in a sample, comprising:-

a solid phase on which is immobilised the analyte or an antibody specific for the analyte;

a reporter composition comprising a thermostable adenylate kinase coupled to an antibody specific for the analyte; and

ADP plus associated reagents for conversion of ADP into ATP by thermostable adenylate kinase.

An optional additional component of the apparatus is a thermolabile ATPase.

The components of the apparatus may be combined into a test kit for determining presence and/or amount of an analyte in a sample.

Testing for formation of ATP may be carried out using a number of conventional means, including formation of colour. Particularly preferred is the use of luciferin/luciferase reagents in combination with calibration curves to determine both presence and amount of analyte. The presence of magnesium ions is usually required for formation of ATP, and further details are provided in the prior art publication GB-A-2304892, the contents of which are incorporated herein by reference.

The present invention has been described in relation to the use of thermostable adenylate kinase. More generally, the invention also provides,

in a third aspect, an assay for determining presence and/or amount of an analyte in a sample, comprising:-

exposing the sample to a detector composition, the detector composition comprising an antibody specific to the analyte coupled to a thermostable enzyme;

isolating (i) detector composition that has specifically bound to analyte from (ii) detector composition that has not specifically bound to analyte;

determining the presence and/or amount of detector composition that has bound to analyte by adding a substrate for the thermostable enzyme;

wherein prior to adding the substrate non-thermostable enzymes are destroyed by application of heat.

The thermostable enzyme is suitably a kinase, and may be selected from pyruvate kinase, adenylate kinase and acetyl kinase. All of these catalyse formation of ATP from ADP and may be used with reagent such as luciferin/luciferase.

The third aspect of the invention also provides apparatus for determining presence and/or amount of analyte in a sample, comprising:-

a solid phase on which is immobilised the analyte or an antibody specific for the analyte;

a reporter composition comprising a thermostable enzyme coupled to an antibody specific for the analyte; and

substrate for the thermostable enzyme.

This aspect of the invention confers the advantage that the signal obtained from the thermostable enzyme is not contaminated by any background signals or background noise that may otherwise be obtained from the action of non-thermostable enzymes on the substrate.

Background signals and/or background noise are thus reduced and possibly even removed entirely. In use of a method of the third aspect of the present invention, an analyte is immobilised on a solid phase, a sample is combined with the solid phase and then the solid phase is washed, the solid phase is exposed to a detector composition including an antibody specific to the analyte coupled to a thermostable enzyme, the solid phase is then again washed, the solid phase is then heated to denature non-thermostable enzymes but so as not to denature the thermostable enzyme of the detector composition, and the amount of thermostable enzyme specifically bound to analyte which itself is specifically bound to the solid phase is determined by adding a substrate for the thermostable enzyme and determining how much product is then obtained. Immobilisation of the analyte can be through use of an analyte-specific antibody immobilised on the solid phase, or by directly binding the analyte to the solid phase.

The present invention is thus suitably employed to investigate the effectiveness of a range of agents with potential for surface cleaning of contaminated surfaces to remove cellular material and PrP. Steel, glass and plastic surfaces can all be investigated to determine whether any one is particularly recalcitrant to cleaning, and PTFE can be used as a control surface for comparative purposes.

Thermostable adenylate kinases may be purified from a number of thermophilic and hyperthermophilic microorganisms using a combination of ion exchange, gel filtration and affinity chromatography. The adenylate

kinases may be cloned and expressed in *E.coli* in plasmid or phage libraries. Direct expression can be screened for (after replica plating) by examining pooled colonies for thermostable adenylate kinase activity by incubation with ADP, followed by ATP bioluminescence assay.

A range of commercially available coupling reagents is available for antibody-adenylate kinase conjugation. Both the antibody and the adenylate kinase can be re-purified by affinity chromatography.

In certain uses of the invention, such as in the case that there is no endogenous adenylate kinase or no microbial contamination of the sample or if the risk of such contamination is removed, it is optional to dispense with the step of removing endogenous adenylate kinase. The method of the invention then comprises specifically associating the analyte with a reporter adenylate kinase, adding ADP and testing for formation of ATP. Preferably, prior to addition of ADP, ATP is substantially removed, for example by the use of an ATPase.

Specific embodiments of the invention are now described.

The assay of the present invention can involve the use of conventional equipment and reagents required for known ATP/AK bioluminescence assays, supplemented by a thermal cycler (widely and inexpensively available for PCR), plus two specific enzymes, a thermolabile ATPase and a thermostable adenylate kinase. To illustrate its application, the specific detection of a microorganism at very low cell density is now described.

**Example 1**

A micro-organism is immobilized onto solid surface by non-specifically binding sample components including the microorganism to the solid phase, treating the solid phase to prevent further non-specific binding thereto and

washing (we use a microtitre well in this case but other known solid phases are suitable, such as a latex bead or a magnetic bead). An antibody specific to the micro-organism and coupled to a thermostable adenylate kinase is introduced and allowed to bind, prior to further washing/recovery.

(In the known AK assay, sensitivity would have been limited by the level of sample concentration possible before levels of background ATP and non-specific AK obscured any signal).

The sample is now heated to about 90°C for about 10 minutes in a cell extraction buffer (in a thermal cycler) to denature any endogenous AK present and release any ATP that may be trapped within the micro-organism. The sample is then cooled to 37°C and a thermolabile ATPase added. The sample is incubated for about 10 minutes to remove the background ATP, then the temperatures is raised to about 90°C to denature the thermolabile ATPase.

Next, ADP is added and the temperature maintained at 90°C so the thermostable adenylate kinase can convert ADP into ATP. This incubation generates ATP exclusively from the thermostable adenylate kinase. The ATP thus generated is then assayed by conventional ATP bioluminescence and is directly proportional to the concentration of the target present.

**Example 2**

A micro-organism is captured by a conventional capture technique, using a specific antibody immobilised onto a solid surface (we use a microtitre well in this case but other known solid phases are suitable, such as a latex bead or a magnetic bead). After washing/recovery, a second antibody specific to the micro-organism and coupled to a thermostable adenylate kinase is introduced and allowed to bind, prior to further washing/recovery.

Thus, the method of Example 1 is repeated but using a microorganism immobilized using antibody.

The invention thus provides method and apparatus for a sensitive capture-type assay.

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**Claims**

1. An assay for an analyte, comprising specifically associating the analyte with a reporter adenylate kinase, adding ADP and testing for formation of ATP wherein, prior to addition of ADP, adenylate kinase other than reporter adenylate kinase is substantially removed.

2. An assay according to Claim 1, wherein the amount of reporter adenylate kinase specifically associated with the analyte is substantially proportional to the amount of analyte.

3. An assay according to Claim 1 comprising inactivating endogenous adenylate kinase in the analyte by heating the analyte.

4. An assay according to any of Claims 1-3 wherein the reporter adenylate kinase is thermostable.

5. An assay according to any of Claims 1-4 wherein formation of ATP is measured using luciferin/luciferase.

6. An assay according to any of Claims 1-5 for determining presence and/or amount of an analyte in a sample, comprising

   exposing the sample to a reporter adenylate kinase coupled to a binding agent specific for the analyte, so that the reporter adenylate kinase is specifically associated with any analyte present in the sample;

   removing reporter adenylate kinase that is not specifically associated with analyte;

exposing reporter adenylate kinase specifically associated with the analyte to ADP; and

testing for formation of ATP,

wherein prior to addition of ADP adenylate kinase other than reporter adenylate kinase is substantially removed.

7. An assay according to any of Claims 1-6 comprising adding an ATPase to the analyte and removing the ATPase from the analyte prior to adding ADP.

8. An assay according to Claim 7 wherein the ATPase is inactivated by heating the ATPase.

9. Apparatus for determining the presence and/or amount of analyte in a sample comprising:-

a solid phase on which is immobilised the analyte or an antibody specific for the analyte;

a reporter composition comprising a thermostable adenylate kinase coupled to an antibody specific for the analyte; and

ADP plus associated reagents for conversion of ADP into ATP by thermostable adenylate kinase.

10. Apparatus according to Claim 9 further comprising an ATPase.

11. An assay for determining presence and/or amount of an analyte in a sample, comprising:-

exposing the sample to a detector composition, the detector composition comprising an antibody specific to the analyte coupled to a thermostable enzyme;

isolating (i) detector composition that has specifically bound to analyte from (ii) detector composition that has not specifically bound to analyte;

determining the presence and/or amount of detector composition that has bound to analyte by adding a substrate for the thermostable enzyme;

wherein prior to adding the substrate non-thermostable enzymes are destroyed by application of heat.

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